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Tumor inhibition by genomically integrated inducible RNAi-cassettes

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ABSTRACT

RNA interference (RNAi) has emerged as a powerful tool to induce loss-of-function phenotypes by post-transcriptional silencing of gene expression. In this study we wondered whether inducible RNAi-cassettes integrated into cellular DNA possess the power to trigger neoplastic growth. For this purpose inducible RNAi vectors containing tetracycline (Tet)-responsive derivatives of the H1 promoter for the conditional expression of short hairpin RNA (shRNA) were used to target human polo-like kinase 1 (Plk1), which is overexpressed in a broad spectrum of human tumors. In the absence of doxycycline (Dox) HeLa clones expressing TetR, that carry the RNAi-cassette stably integrated, exhibited no significant alteration in Plk1 expression levels. In contrast, exposure to Dox led to marked downregulation of Plk1 mRNA to 3% and Plk1 protein to 14% in cell culture compared to mismatch shRNA/Plk1-expressing cells. As a result of Plk1 depletion cell proliferation decreased to 17%. Furthermore, for harnessing RNAi for silencing disease-related genes *in vivo* we transplanted inducible RNAi-HeLa cells onto nude mice. After administration of Dox knockdown of Plk1 expression was observed correlating to a significant inhibition of tumor growth. Taken together, our data revealed that genomically integrated RNAi-elements are suitable to hamper tumor growth by conditional expression of shRNA.

INTRODUCTION

RNA interference (RNAi) is a powerful tool for post-transcriptional downregulation of endogenous genes. Small double-stranded RNAs named short interfering RNAs (siRNAs) or micro RNAs (miRNAs) depending on their origin abrogate gene expression by sequence-specific binding of cognitive mRNA. In the first case binding of siRNA evokes mRNA elimination by RNAi whereas binding of miRNA results in mRNA translation arrest (1,2). For transient gene silencing numerous investigators administered synthetic small double-stranded siRNAs with 2 nt 3′-overhangs to mammalian cells (3,4). Alternative attempts for prolonged RNAi-based gene silencing have been made through application of Pol III (RNA polymerase III)-promoter driven expression of short hairpin RNAs (shRNAs) that are subsequently processed to siRNAs (5–8). In this regard most commonly used Pol III-dependent transcriptional units are the promoters for U6 snRNA and for H1 RNA. The use of Pol III promoters offers two important advantages: (i) a high transcription rate of $\sim$1–4 $\times$ 10^5 transcripts per cell, (ii) no additional nucleotides are added to the transcript allowing for optimal functional activity of shRNAs. The structures of Pol III promoters fall into three classes. One of these, representing type III genes, encompasses the H1 promoter which consists of four *cis*-acting elements lying in the 5′-flanking sequence very closely together in a region of 100 bp in length: the TATA box (positions -26 to -31 relative to the transcription start site), the PSE (proximal sequence element; positions -51 to -68) and the DSE (distal sequence element; positions -69 to -100) containing a staf binding site and an octamer motif (5,9). Although the U6 snRNA promoter, which belongs also to type III of the Pol III promoter family, exhibits a distance of $\sim$150 bp between PSE and DSE, the regulatory units (PSE and staf binding site of DSE) are adjacent in the H1 promoter. Both, staf binding site and octamer motif, as components of the DSE, contribute to full promoter activity. Additionally, PSE- and DSE-binding factors seem to interact (10–12).

A major obstacle for the generation of loss-of-function mutants is the constitutive expression of shRNA when targeted genes are essential for cell survival. Consequently many attempts were made to develop inducible promoters for the expression of shRNA dependent on inducers like tetracycline (13,14), steroid hormones (15), heat shock proteins (16) or using the Cre-LoxP system (17). Restrictions in versatility of these promoters are caused by their leakiness in the uninduced state and are partially due to pleiotropic effects which occur by inducing mechanisms themselves.

We identified and cloned the human serine/threonine kinase polo-like kinase 1 (Plk1) and demonstrated its

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overexpression in multiple types of tumors (18). Plk1 is evolutionally highly conserved and plays a leading role for numerous aspects of mitotic progression including G_2/M-transition (19), centrosome maturation (20,21), mitotic spindle formation (22) and activation of the anaphase-promoting complex (23,24). Plk1 is activated by phosphorylation at the G_2/M-phase boundary. *In vivo* phosphorylation of Plk1 at Ser-137 and Thr-210, which is located in the activation loop of the kinase domain, occurs in mitosis (25). Levels of Plk1 mRNA and protein are massively elevated in many kinds of tumors like breast, ovarian, non-small cell lung cancer and melanomas (18,26–29). High Plk1 expression often correlates to poor prognosis of cancer patients (29,30) Additionally, overexpression of Plk1 induces transformation in NIH/3T3 fibroblasts and tumor progression in nude mice (31). Interestingly, inhibition of Plk1 expression in cancer cells via application of antisense oligonucleotides, siRNA or vector-mediated expression of shRNA resulted in mitotic arrest and induction of apoptosis *in vitro* and *in vivo* (4,32,33). Increasing evidence suggests that Plk1 represents an ideal target for cancer drug development (34).

In this study we investigated whether inducible RNAi cassettes expressing shRNA against Plk1 integrated into the genome are suitable genetic elements to protect mammalian cells against neoplastic proliferation. For this purpose we used Tet-inducible derivatives of the H1 promoter driving the expression of shRNA targeting the mRNA of Plk1. Following genomic integration we studied the tetracycline-dependent kinetics of activity of different inducible promoter variants reflected by the conditional downregulation of Plk1 in HeLa cells. Analysis of quantitative properties such as leakiness under non-induced conditions and full activity in the on-state supported the functional evaluation of the novel regulatory systems. In addition, the conditional knockdown of Plk1 in cultured cells and in xenografted tumor mouse models helped to answer the question whether the genomic integration of inducible shRNA/Plk1 cassettes bears the potential to counteract tumor cell proliferation in a conditional manner.

MATERIALS AND METHODS

Genetic constructs for the expression of shRNAs targeting Plk1

Three types of expression plasmids (i) ptet O-T-hH1/shRNAPlk1 (pUS); (ii) ptet T-O-hH1/shRNAPlk1 (pDS) and (iii) ptet O-T-O-hH1/shRNAPlk1 (pUS/DS) containing different variants of Tetracycline-inducible H1 promoters encoding shRNA targeting Plk1 were generated as described previously (14). The targeting sequence in human Plk1 (GenBank accession no. NM_005030) is GTGCTTCGAGATCTCGGAC corresponding to the coding region 198–216 relative to the first nucleotide of the start codon. The plasmid phH1/shRNAPlk1MM (pwtH1MM) containing the wild-type H1 promoter expressing a mismatched shRNA (5′-GUGCACUGAGAUCUCGGACUU) was used for the generation of stable clones as constitutive control. Furthermore, the plasmids pUS, pDS and pUS/DS expressing also the mismatched shRNA (GUGCACUGAGAUCUCGGACUU) were used for the generation of stable clones as inducible control.

Generation of stable cell clones

T-REx™-HeLa cells stably expressing bacterial TetR were obtained from Invitrogen GmbH (Karlsruhe, Germany) and cultured in MEM with Earle's and GlutaMAX™ (Invitrogen GmbH) containing 10% Tet system approved fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), penicillin-streptomycin (Invitrogen GmbH) and 5 μg/ml blasticidin (Invitrogen GmbH).

T-REx™-HeLa cells were transfected with the above listed RNAi-plasmids pUS, pDS, pUS/DS for stable integration and subsequent inducible expression of shRNA/Plk1 or shRNA/Plk1MM using Fugene 6 (Roche Diagnostics, Mannheim, Germany) as transfection reagent according to the manufacturer's instructions. In addition, pwtH1MM was transfected under the same conditions. Transfected cells were selected by using culture medium containing 1.5 mg/ml geneticin (G418; Invitrogen GmbH) starting 24 h after transfection. Altogether selection of resistant cells lasted for a period of 25 days. Subsequently geneticin-resistant cells were trypsinized, centrifuged and resuspended in culture medium. Cell numbers were determined using a hemacytometer. Selected cells were diluted to obtain 1 cell/200 μl, plated out onto 96-well plates and cultivated without antibiotic until a cell number of 5×10^5 was reached. Cell clones were expanded under continuous selection using 500 μg/ml geneticin (G418; Invitrogen GmbH).

PCR, Southern blot analysis and preparation of genomic DNA

To test whether resistant clones contain integrated plasmid DNA including different promoter variants including the shRNA-coding region, genomic DNA was prepared using a DNA Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To gain first evidence for complete integration of inducible RNAi-cassettes (complete promoter including shRNA-coding sequence), we performed PCR-based controls. As sense primer we used 5′-CGCCCTGCAATATTTGCATG-3′ binding the H1 promoter variants at a 5′ position. As antisense-primer we used 5′-CCGTCGACTGCAGAATTCGAAGC-3′ which binds to a plasmid-derived sequence 3′ of shRNA-coding stretch. Genomic DNA from non-transfected HeLa cells (negative) and plasmid DNA (positive) were used as controls.

To examine the copy number of promoter cassettes integrated into the genome of different cell clones, a Southern blot analysis using genomic DNA digested with HindIII was performed as described previously (35). Integrated RNAi-cassettes were detected with an [α-^{32}P]-labeled probe generated using the following reaction mixture: 100–200 μCi of [α-^{32}P]dCTP (6000 Ci/mmol), the three other dNTPs (each at 50 μM) and 200 nM of the primers 5′-GCGGACAGGTATCCGGTAAG-3′ (sense) and 5′-CCGTCGACTGCAGAATTCGAAGC-3′ (antisense).

Cell culture and induction of shRNA expression following addition of doxycycline

Positive cell clones with stably integrated, inducible RNAi-cassettes were cultured as described above. During the analysis of shRNA-based gene silencing culture medium contained geneticin (G418; Invitrogen) at a concentration of

500 μg/ml. Expression of shRNA by conditional promoters was induced through the addition of Dox (doxycycline hydrochloride; Sigma, Taufkirchen, Germany) at different concentrations (1–10 μg/ml) beginning simultaneously with cell seeding. Dox-containing medium was replaced every 24 h. Each experiment was performed at least five times.

Cell lysis and Western blot analysis

At 120 h after induction with Dox, cell extracts and Western blots were prepared as described previously (4,36). Membranes were incubated with monoclonal antibodies against Plk1 (1:300; Biomol, Hamburg, Germany), polyclonal antibodies against β-actin (1:200 000; Sigma) and with goat anti-mouse serum (1:2000; Santa Cruz Biotechnology Inc., Heidelberg, Germany). Immunocomplexes were visualized as described previously (4).

Preparation of RNA and Northern blot analysis

Total RNA was isolated using RNeasy Mini Kit according to the manufacturer's protocol (Qiagen), 120 h after induction with Dox. Probes for Northern blots were generated by radiolabeling antisense strands of Plk1 as described previously (4). Northern blotting, hybridization and standardization were carried out as described previously (35).

Determination of cell proliferation

The growth rate of 1.5×10^5 cells was determined by counting cells using a hemacytometer at 24, 48, 72, 96 and 120 h after beginning of induction. Wild-type HeLa cells were treated with 5 μg/ml Dox to test its effect on cell proliferation.

Xenografted tumor mouse model

Each flank of 8-week-old male athymic mice (Harlan-Winkelmann, Borchen, Germany) was inoculated with 1×10^7 stably transfected T-Rex-HeLa cells resuspended in 0.5 ml sterile saline solution subcutaneously. HeLa clones for inducible expression of shRNA/Plk1MM were inoculated on the left flank whereas clones for inducible expression of shRNA/Plk1 were inoculated on the right flank. Mice were distributed randomly into two groups (12 animals each) for the subsequent analysis of tumor development with and without Dox-treatment. Dox (Sigma) was applied as 0.2% solution via the drinking water with additional 3% sucrose dissolved in natural mineral water starting 2 days after inoculation. Drinking water for control mice contained 3% sucrose without Dox. Xenografted tumors were measured in two right-angled dimensions by a sliding caliper twice a week. Absolute tumor volumes were evaluated according to $V\ (\mathrm{mm}^3) = \pi/6\ ab^2$ with $a > b$. Relative tumor volumes represent the ratio of current tumor volume and corresponding volume at the beginning of the study. After inoculation measurements started at day 23 and finished at day 51 followed immediately by sacrificing the animals. Eight individual tumors from the Dox-treated as well as from the untreated groups were analyzed. Tumor mRNA was prepared as described previously (35).

Statistical methods

All experiments were performed at least in triplicate. Standardization and statistics were performed as described previously (4). Paired *T*-tests (two-sided) were performed for all experiments. Significant reductions ($P < 0.05$) were indicated with an asterisk.

RESULTS

Generation of HeLa cell lines carrying RNAi-cassettes for the conditional expression of shRNA targeted to Plk1

Different H1 promoter variants were used for the generation of stable cell lines carrying inducible RNAi-cassettes as integral component of chromosomal DNA. A palindromic operator sequence from the tet operon control region, called TetO (37) for the occupation by the TetR was inserted adjacent to the TATA box (Figure 1A): (i) In the US-type promoter the TetO was inserted upstream of the TATA box and substituted completely the sequence connecting PSE element and TATA box; (ii) in the DS-type the TetO was inserted downstream of the TATA box and partially replaced the sequence between TATA box and the initiation site for transcription of the H1 RNA; (iii) the US/DS-type contains two TetO elements located 5′ and 3′ connected to the TATA box (14). In all constructs the distance found in the wild-type H1 promoter between the PSE and transcription start site was kept constant. To examine the response of different H1 promoter constructs, as elements integrated into the genome of HeLa cells, to an effector, we studied the inhibition of Plk1 expression by conditional expression of shRNA/Plk1 driven by these promoters. For this purpose we generated RNAi-cassettes and linked all H1 promoter constructs to a 19 bp Plk1-derived sequence that, when transcribed, forms a shRNA containing a 19 bp stem and a 9 bp loop which is likely processed to siRNA. A termination sequence was included which consists of five thymidine nucleotides. Two uracil nucleotides are predicted to constitute the 3′ end of the corresponding shRNA. Vectors for the expression of Plk1-specific shRNA or a corresponding mismatched sequence used for the integration as stable RNAi-cassettes were named according to the location of TetO sequence(s): pUS (upstream), pDS (downstream) and pUS/DS (upstream/downstream). In addition, a construct was generated containing the wild-type H1 promoter constitutively driving the expression of a mismatched Plk1 sequence (phH1/shRNAPlk1MM) as control.

T-REx™-HeLa cells stably expressing bacterial TetR were transfected with plasmids containing different Tet-responsive RNAi-cassettes or a plasmid containing the wild-type H1 promoter constitutively expressing the mismatched sequence (Figure 1).

After clonal selection cells were analyzed for the genomic integration of the above described RNAi-cassettes. A PCR-based analysis disclosed the existence of the above described H1 promoter elements driving the expression of shRNA/Plk1 or shRNA/Plk1MM in the genomes of transfected cells (Figure 2A). Subsequent sequencing of the PCR products confirmed the integrity of the promoter constructs encompassing corresponding shRNA-sequences. Furthermore we investigated the number of integrated copies of elements. A Southern blot analysis revealed two integration sites for the US clone (Figure 2B, lane 2) whereas the DS and US/DS

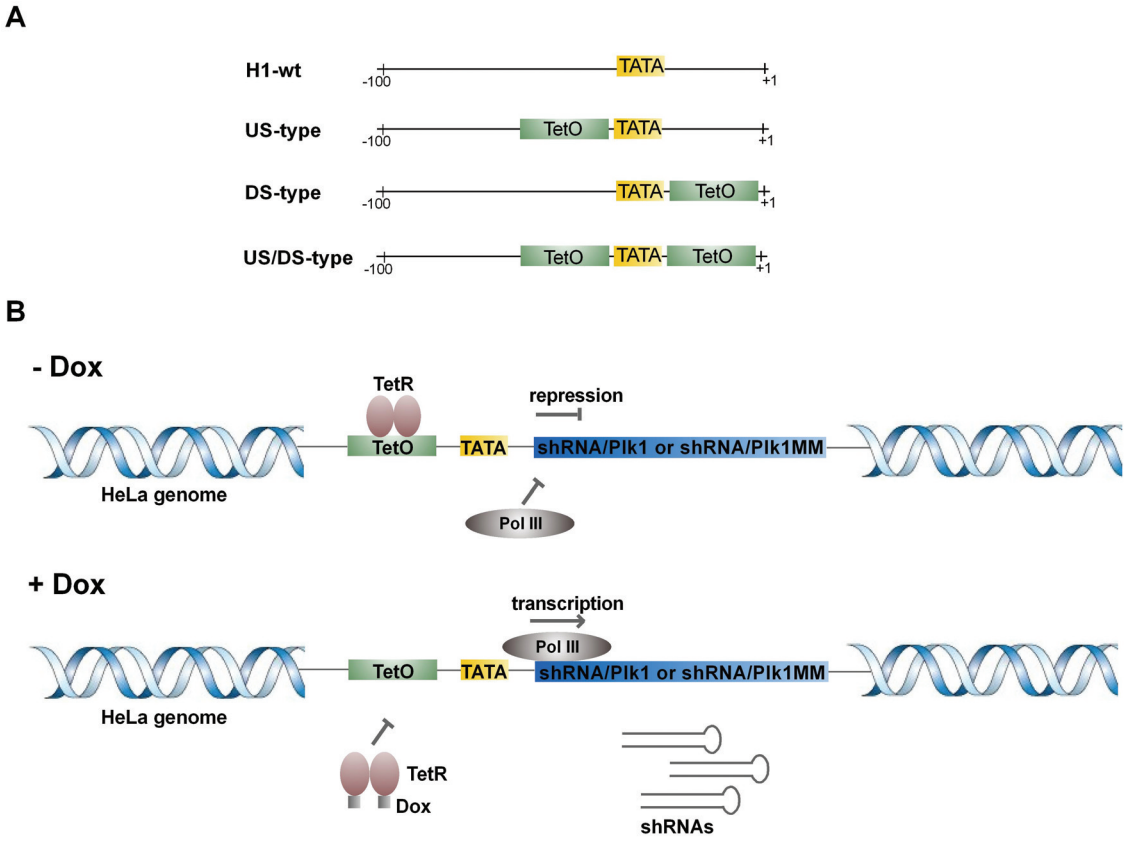


Figure 1. Genomically integrated cassettes for constitutive or Tet-inducible expression of shRNA/Plk1 or shRNA/Plk1MM. (**A**) Different promoter derivatives for constitutive or Tet-inducible expression of shRNA/Plk1 or shRNA/Plk1MM were stably integrated into TetR-expressing HeLa cells followed by isolation of single cell clones: wild-type H1 promoter for constitutive expression of mismatch shRNA, US-type promoter containing TetO upstream of the TATA box; DS-type promoter containing TetO downstream of the TATA box and the US/DS-type promoter containing TetO upstream and TetO downstream of the TATA box. TetO, tetracycline operon; TATA, TATA box. (**B**) Schematic representation of promoter function in absence (−Dox) and presence of doxycycline (+Dox).

clones both exhibited one integration site (Figure 2B, lanes 3 and 4).

Dox-dependent reduction of cellular Plk1 expression using different Tet-responsive H1 promoter variants

We investigated the dose-response for Dox, a tetracycline derivative, which was shown previously to be a very efficient effector for TetR (38). In the absence of the synthetic regulator Dox TetR binds to its operator and prevents expression of the shRNA transcripts by RNA polymerase III (Figure 1B). To evaluate the response of different promoter constructs to Dox, at first Plk1 mRNA levels of different cell clones (US, DS and US/DS) were analyzed by Northern blotting. We chose elevated concentrations of Dox ranging from 1 to 10 μg/ml for efficient induction of gene expression, because TetR is highly expressed under the control of a constitutive CMV promoter. Plk1 mRNA was reduced to levels of 3% ($P < 1 \times 10^{-4}$) by shRNA/Plk1 driven by the US clone, to levels of 3% ($P = 3 \times 10^{-4}$) by the DS clone and to levels of 9% ($P = 0.002$) by the US/DS clone compared to the Plk1 mRNA level in the clone constitutively expressing the mismatched sequence (Figure 3A, lanes 1, 5, 9 and 13), 120 h after induction with 10 μg/ml Dox. To investigate whether the reduction of Plk1 mRNA levels is specific and correlates to the sequence of the Plk1 shRNA, we generated clones which express a mismatched sequence under the control of all three promoter types. All clones were tested for the presence of the correct promoter and shRNA-coding sequences. We repeated the expression analysis under the same conditions as described above. A significant alteration of the endogenous Plk1 level was not observed using a mismatched shRNA sequence driven by the US, DS or US/DS promoter (Figure 3A, lanes 14–22). Thus, downregulation of the endogenous Plk1 transcript levels seems to be due to an RNAi-mediated mechanism targeting the Plk1 transcript. Although in the absence of Dox the US and US/DS promoters showed no leakiness, the mRNA level of Plk1 was reduced to levels to 65% by the DS promoter in the off-state (Figure 3A, lanes 2, 6 and 10).

Just like all three cell clones containing different promoter types driving the expression of shRNA targeting Plk1 showed a significant downregulation of Plk1 mRNA in response to increasing concentrations of Dox, we wondered whether silencing of endogenous transcription via expression of shRNA/Plk1 also translates to altered Plk1 protein levels. The US clone exhibited a low level of Plk1 protein which was significantly reduced to 14% ($P < 1 \times 10^{-4}$) (Figure 3B, lane 5) compared to the clone constitutively driving the expression of shRNA/Plk1MM (MM clone) (Figure 3B, lane 1), 120 h after induction with Dox at a concentration of 10 μg/ml. The MM clone showed an

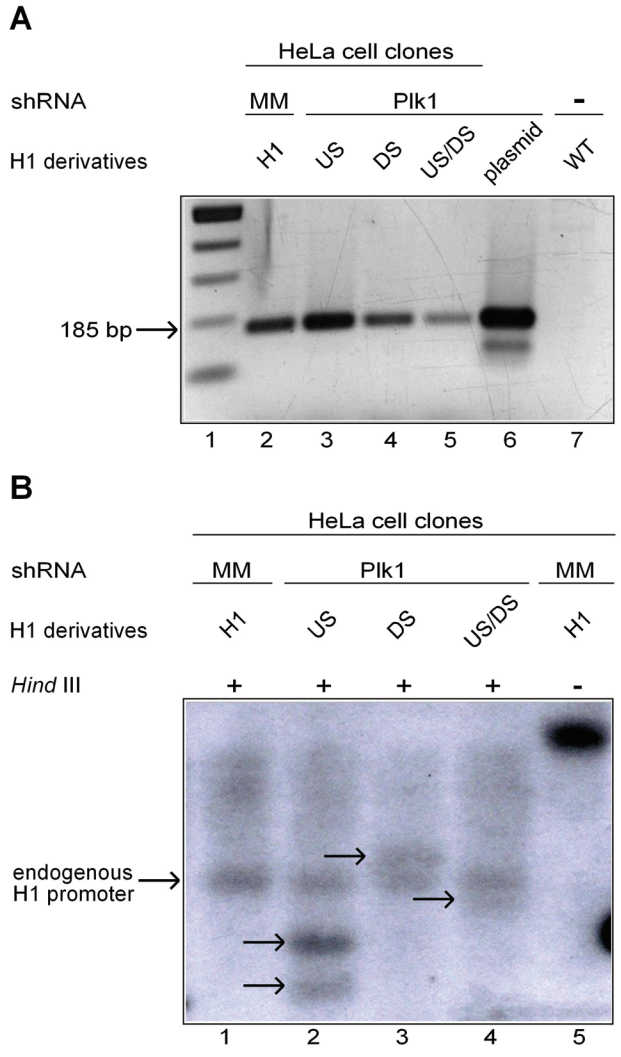


Figure 2. Detection of genomically integrated RNAi-cassettes in HeLa cell clones. Genomic DNA from HeLa cell clones digested with HindIII was further analyzed. (**A**) PCR was performed to amplify a sequence of 185 bp in length enclosing different H1 promoter derivatives and shRNA-coding regions. Subsequent sequencing of amplified fragments revealed the authentical integration of different RNAi-cassettes. DNA from wild-type HeLa cells was used as negative control (lane 7), recombinant plasmid DNA was used as positive control (lane 6). (**B**) Southern blot analysis was performed to determine the number of integrated RNAi-cassettes (arrows). A radioactive probe specific for RNAi-cassettes was used.

equal Plk1 protein level compared to wild-type HeLa cells (data not shown) suggesting the specificity of our RNAi-based approach. In the absence of Dox the US clone exhibited a Plk1 protein level of 92% compared to the level of HeLa cells expressing the mismatch shRNA (MM) under the control of the parental H1 promoter (Figure 3B, lanes 1 and 2). The expression of mismatched shRNA under the control of all three inducible promoters did not influence endogenous Plk1 protein levels significantly either (Figure 3B, lanes 14–22). The analysis indicates a very broad range of Plk1 regulation for the US clone under different Dox conditions.

At a concentration of 10 μg/ml the US/DS clone showed nearly similar properties compared with the US clone; although in the non-induced state Plk1 protein levels were found to be 95% compared to control cells (Figure 3B, lane 10), a significant downregulation in the induced state was observed (15% Plk1 protein, $P = 1 \times 10^{-4}$) (Figure 3B, lane 13). At a concentration of 1 μg/ml Dox the US and the US/DS clone responded differently; whereas US/DS clone exhibited no significant downregulation of the Plk1 protein compared to the non-induced state (90%, $P > 0.5$) (Figure 3B, lane 11), pUS clone differed clearly by showing a significantly reduced Plk1 protein level (67%, $P < 0.05$) (Figure 3B, lane 3).

The DS clone also showed a significant downregulation of Plk1 protein in the presence of 10 μg/ml Dox (15%, $P = 1 \times 10^{-4}$) (Figure 3B, lane 9). A limited extent of leakiness could be observed in the non-induced state (81% Plk1 protein) (Figure 3B, lane 6). Thus, by using the US clone or the US/DS clone for the expression of shRNA/Plk1, transcript and protein levels of Plk1 could be triggered in a wider range as seen for the DS clone.

Doxycycline-triggered downregulation of Plk1 leads to the inhibition of cancer cell proliferation

Numerous studies demonstrated the limited duration of gene silencing activity by the application of siRNA or by transient transfection of shRNA-expressing vectors. This is a major disadvantage of exogenously administered RNAi-inducing agents. Because of this reason these techniques led only to a transient inhibition of cancer cell proliferation through silencing of Plk1 (4,14,33,35,39). To prolong the RNAi-based effects on Plk1-function, RNAi-cassettes were integrated into the genome of TetR-expressing HeLa cells and tested for their capability to trigger cancer cell proliferation. For this purpose all four established cell clones were cultivated with or without Dox.

US cells showed the highest proliferation rate of 78% compared to wild-type HeLa cells under non-induced conditions, 120 h after seeding (Figure 4A). Cells derived from the DS clone or from the US/DS clone exhibited in the absence of Dox reduced proliferative activity of 54 and 68%, respectively, compared to wild-type HeLa cells (Figure 4B and C). In contrast, after addition of 5 μg/ml Dox cell proliferation of all three clones was significantly inhibited compared to the non-induced state: US clone to 33% ($P = 3 \times 10^{-4}$), DS clone to 20% ($P = 6 \times 10^{-4}$) and the US/DS clone to 17% ($P = 2 \times 10^{-4}$) (Figure 4A–C). To evaluate whether the effect of proliferative inhibition is influenced by the addition of Dox alone, wild-type HeLa cells were cultivated at a Dox concentration of 5 μg/ml for 120 h. Wild-type HeLa cells showed no significant alteration in proliferative activity compared to HeLa cells grown in the presence of Dox ($P > 0.5$) (Figure 4A–C). Mismatched shRNA expression driven by all three promoter types did not alter cell proliferation with or without Dox significantly compared to the growth rate of control cells (Figure 4A–C).

Conditional RNAi-mediated silencing of Plk1 reduces tumor growth in xenografted mice

Tetracycline-inducible expression systems have been widely used in mouse models for studying gene function (40). To test whether inducible gene silencing driven by

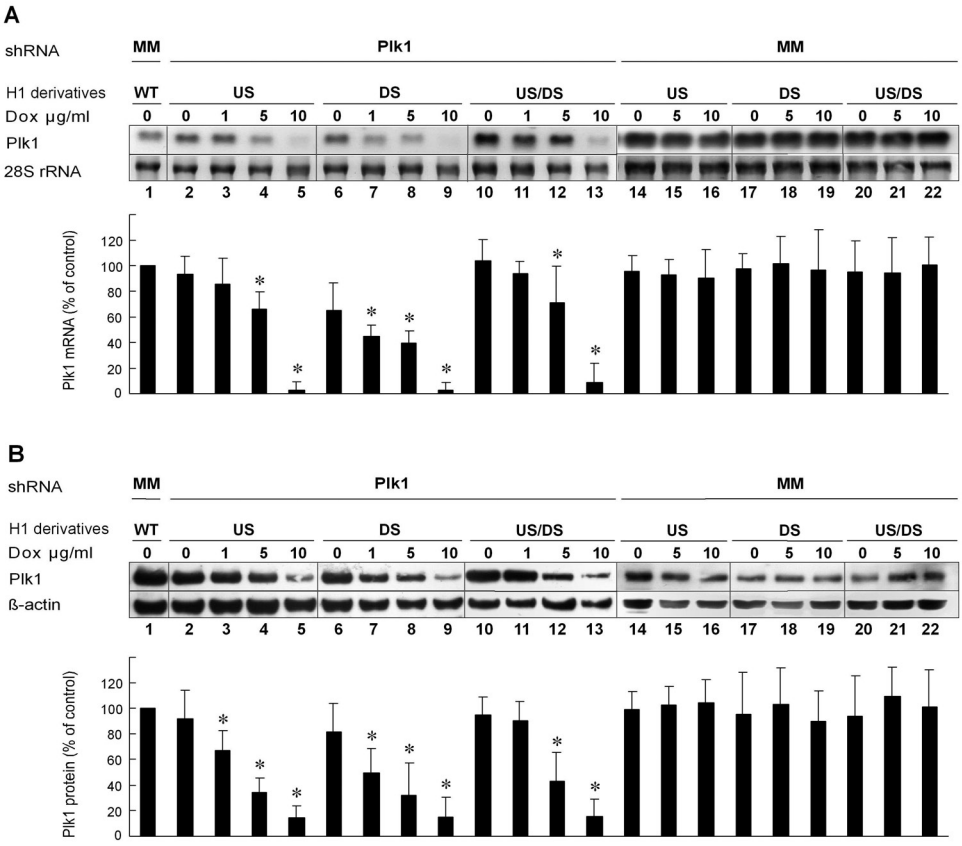


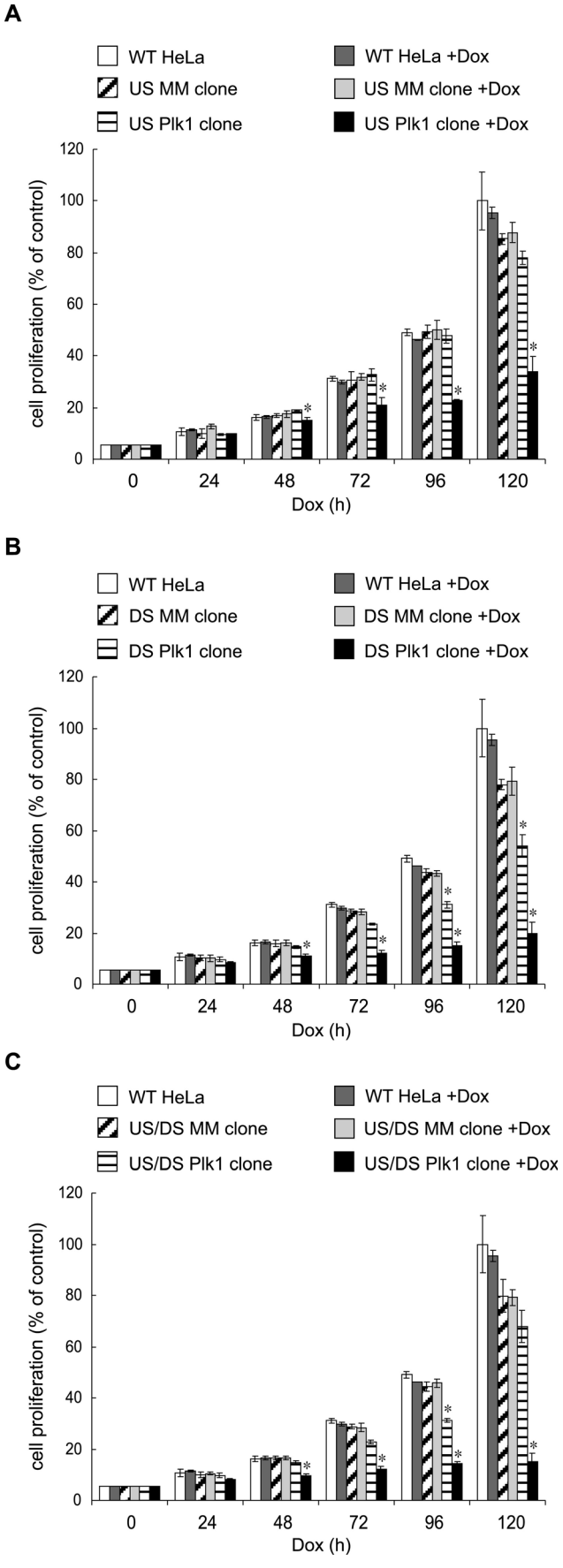
Figure 3. Knockdown of Plk1 mRNA and protein by Tet-inducible expression of shRNA/Plk1 using different promoter types stably integrated into the genome of HeLa cell clones. (**A**) Northern blot analysis of Plk1 mRNA. HeLa cell clones containing a wild-type H1 promoter constitutively expressing shRNA coding for a mismatch sequence (MM) was used as control (lane 1); Plk1 mRNA expression in Tet-inducible US-type promoter-derived cell clones driving the expression of shRNA/Plk1 (lanes 2–5); Plk1 mRNA expression in Tet-inducible DS-type promoter-derived cell clones driving the expression of shRNA/Plk1 (lanes 6–9); Plk1 mRNA expression in Tet-inducible US/DS-type promoter-derived cell clones driving the expression of shRNA/Plk1 (lanes 10–13). Plk1 mRNA expression in Tet-inducible US-, DS- and US/DS-type promoter-derived cell clones driving the expression of shRNA/Plk1MM (14–22). Administration of different Dox concentrations lasted for 120 h. For standardization ethidiumbromide staining of 28S rRNA was chosen. Plk1 mRNA levels were presented as percentage of Plk1 mRNA levels in cell clones constitutively expressing mismatched shRNA. (**B**) Immunoblot analysis of Plk1 protein expression. Same distribution of samples as shown in (A). For standardization β-actin expression was used. Plk1 protein levels were presented as percentage of Plk1 protein levels in cell clones constitutively expressing mismatched shRNA. Significant reductions ($P < 0.05$) are indicated with an asterisk.

genomically integrated RNAi-cassettes as studied in cell culture possesses the power to inhibit neoplastic growth in xenograft models, we inoculated 8-week-old immunodeficient athymic nude mice with different cell clones containing the US and the US mismatched RNAi-cassettes. The injection of the US clone on one flank and the corresponding US mismatched clone on the other flank provided a side-by-side comparison in the presence or absence of Dox. Cells (1×10^7) were injected subcutanously into each flank of nude mice. The relative tumor growth of xenografted conditional RNAi-tumors analyzed for 28 days terminating 51 days post inoculation is depicted in Figure 5A. Mice inoculated with different US cell clones showed a downregulation of tumor growth after induction. If 0.2% Dox was added to the drinking water of tumor-bearing mice, US clone-derived tumors exhibited a significant reduction to 47% ($P = 0.006$) 51 days after inoculation compared to the corresponding mismatched shRNA-expressing clone (Figure 5A). The growth behavior of the non-induced US clone, the induced and non-induced US mismatch clone did not differ significantly ($P > 0.5$) (Figure 5A).

In order to examine whether reduced tumor growth correlates with silencing of Plk1 expression, total mRNA from US tumors and US mismatched tumors derived from mice treated with or without Dox were subjected to a Northern blot analysis. Consistent with the findings of reduced US tumor growth for mice treated with Dox, the average Plk1 mRNA levels of respective mice were reduced to 73% ($P = 0.034$) (Figure 5B) compared to control mice that express mismatched shRNA with Dox-treatment (Figure 5B).

DISCUSSION

In our study expression of shRNA was driven by a conditional tetracycline-dependent H1 promoter integrated into the genome of TetR- expressing HeLa cells. This system was used to downregulate the expression of endogenous Plk1 aiming at the inhibition of cancer cell proliferation *in vitro* and in a xenograft mouse model. Although shRNA/Plk1 under the control of different H1 promoter variants in the absence of Dox influenced the expression of Plk1 only



in a very limited fashion due to varying leakiness, the induction of shRNA/Plk1 expression 120 h post administration of Dox led to significantly decreased mRNA and protein levels. These effects result in a distinct inhibition of cancer cell proliferation *in vitro* and *in vivo*.

A Pol III-based promoter using the prokaryotic TetR-TetO system has been used originally to control the expression of eukaryotic tRNA in *Saccharomyces cerevisiae* (41). In recent years many approaches tested the TetO-TetR system for conditional expression of different RNAs in mammalian cells (42–44). In this context especially the U6 promoter (42) and the H1 promoter (14) were analyzed structurally and functionally in detail. Still, the use of the U6 promoter implicates some disadvantages: the cellular response to the introduction of increased active U6 promoter is not an increase of endogenous transcriptional activity but rather an increase of U6 transcript degradation (45). This response describes a so far unrecognized U6-specific intracellular negative feedback mechanism through which accumulation of U6 snRNA is counteracted. Owing to this observation which is a major obstacle to guarantee high expression rates, we decided to investigate the properties of different inducible H1 promoter variants. We studied the functional consequences of varying TetO localizations upstream of the transcription start site within the H1 promoter for the conditional expression of shRNA/Plk1 as element integrated into the genome of HeLa cells. Different positioning of the TetO sequence showed considerable differences for the activity of the H1 promoter variants in the presence or absence of Dox; whereas stably integrated into the genome both, US-type and US/DS-type, promoters exhibited low leakiness (93–104% Plk1 mRNA; 92–95% Plk1 protein), the DS-type promoter showed higher basal activity in the non-induced state as reflected by lower cellular Plk1 mRNA (65%) and protein levels (81%). Application of Dox resulted in a strong reduction of Plk1 mRNA (3–9%) and protein levels (14–15%) independent of the promoter derivative used. The low leakiness of the US promoter might be due to the binding of the Tet repressor to a location upstream of the TATA box which plays a major role for the transcriptional activity of the H1 promoter (9). In contrast, alterations of the sequence downstream of the TATA box as shown by Myslinski *et al*. (9) or binding of the Tet repressor to this region does not seem to have a major inhibitory effect on the transcriptional activity of the H1 promoter.

A difference to previous transient transfection experiments was found for the non-induced state: the leakiness of the DS- and US/DS-type promoter was less pronounced in the stable situation compared to the transient state (14). This quantitative difference of the transcriptional readout might be due

Figure 4. Proliferative activity of inducible cell clones expressing shRNA/Plk1MM or shRNA/Plk1. HeLa cell clones containing stably integrated H1 promoter derivatives were induced for the expression of shRNA/Plk1MM or shRNA/Plk1MM by addition of 5 μg/ml Dox to the culture medium. (**A**) Proliferative activity of cell clones containing promoter of the US-type for the expression of shRNA/Plk1MM or shRNA/Plk1; (**B**) proliferative activity of cell clones containing promoter of the DS-type for the expression of shRNA/Plk1MM or shRNA/Plk1; (**C**) proliferative activity of cell clones containing promoter of the US/DS-type for the expression of shRNA/Plk1MM or shRNA/Plk1. Values are shown in percent compared to wild-type HeLa cells. Significant growth inhibitions ($P < 0.05$) are indicated with an asterisk.

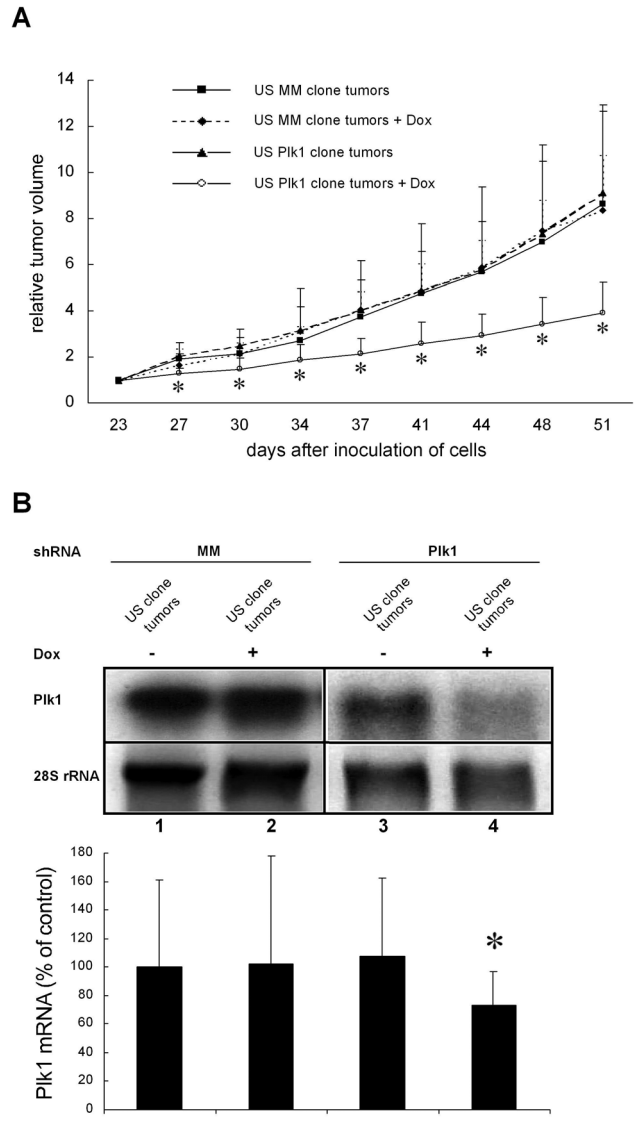


Figure 5. Tumor growth in nude mice after Dox-induced expression of shRNA/Plk1MM or shRNA/Plk1. Mice were inoculated with 1×10^7 HeLa cell clones in each flank HeLa clones for inducible expression of shRNA/Plk1MM were inoculated on the left flank whereas clones for inducible expression of shRNA/Plk1 were inoculated on the right flank. (**A**) Relative tumor volumes of xenografted US-type HeLa tumors for inducible expression of shRNA/Plk1MM or shRNA/Plk1 either after induction with Dox or in the absence of Dox starting 23 days after inoculation. Significant inhibition of tumor growth ($P < 0.05$) is indicated with an asterisk. (**B**) Northern blot analysis of Plk1 mRNA under the influence of shRNA/Plk1MM or shRNA/Plk1 expression. Plk1 mRNA expression in US-type promoter-derived HeLa tumors of mice without Dox-treatment (lanes 1 and 3) in comparison to tumors of mice treated with Dox (lanes 2 and 4). For standardization ethidiumbromide staining of 28S rRNA was chosen. Significant reduction ($P < 0.05$) is indicated with an asterisk.

to an elevated copy number of plasmids in the transient situation compared to only one or two copies of the RNAi-cassette per genome found in this study. Single RNAi-cassettes seem to be sufficient to knockdown endogenous Plk1 mRNA to levels of 3–9% at a concentration of 10 μg/ml Dox. In contrast, a multitude of copies in the transient situation (14) does not seem to augment the silencing effect compared to the impact of two integrated copies analyzed in this study under induced conditions but rather seem to enhance unwanted leakiness in the non-induced state.

In addition to the functional characterization of genomically integrated RNAi-cassettes targeting the human Plk1 gene in cell culture, we wondered whether this approach is suited for efficient gene silencing *in vivo* using a xenograft mouse model. Hitherto in only a few studies conditional tetracycline-dependent RNAi-systems were applied: (i) Although the conditional knockdown of the PI 3-kinase in an orthotopic prostate cancer mouse model failed to inhibit tumor growth in nude mice, it reduced invasive tumor cell growth (formation of metastasis) (46). (ii) The candidate tumor suppressor KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor for the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is a promising agent for cancer therapy. However, its contribution to the inhibition of tumor growth and its role as a determinant of chemosensitivity are poorly understood. Inducible silencing of KILLER/DR5 *in vivo* by exposure of mice to Dox led to accelerated growth of bioluminescent tumor xenografts and conferred resistance to the chemotherapeutic agent 5-fluorouracil (47). Recently, shRNA-based gene silencing of hypoxia-inducible factor-1α, oncogenic BRAF or the Met receptor was tested in different xenograft models using nude mice (48–50). These studies demonstrated that inducible RNAi is a versatile tool to validate tumor targets *in vivo*. Our study shows a downregulation of tumor growth by conditional Dox-dependent expression of shRNA/Plk1 driven by genomically integrated RNAi-elements. Still, knockdown of Plk1 in murine tumors was not as efficient as in cell culture where a selective pressure by G418 was maintained during the entire experiment. We cannot exclude that cell clones transplanted onto nude mice lost partially RNAi-cassettes during the long period of tumorigenesis which took 51 days. To enhance the observed inhibitory effect, in future experiments clones harboring additional copies of the inducible RNAi-cassette could be used for prolonged and more intense Plk1 silencing.

Plk1 is a key element of mitotic progression. Plk1 is overexpressed and has prognostic potential in cancer. Accumulating evidence highlights its potential as therapeutic target (34). Multiple studies analyzed the downregulation of Plk1 by antibodies, dominant-negative forms, antisense oligonucleotides, chemically synthesized siRNA and vector-driven expression of shRNAs (4,20,32,35,51). Since the downregulation of Plk1 in all previous experiments was transient, several important issues could not be addressed: (i) Depending on the dose and duration of Plk1 silencing, it is important to monitor the effects in cancer and normal cells; (ii) Mechanisms involved in cell signaling leading to apoptosis by Plk1-inhibitors are still unknown; (iii) How does the mitotic delay caused by Plk1 silencing correlate with the apoptotic response? These questions can hardly be answered under transient conditions, which often include unwanted side-effects caused by transfection-based disturbances of the analyzed cells. Taken together, a stable cell system that allows the regulation of Plk1 activity could be a versatile tool to define its role during the cell cycle more precisely. Furthermore, experience with the long-term RNAi-based modulation of gene expression is limited. Thus, in addition to a better understanding of the mitotic

regulation using our cell system we expect to learn more about the cellular response to prolonged RNAi-based gene silencing, which is an important issue for RNAi-based inhibitors on their way to clinical applications. In summary, our data indicate that inducible tetracycline-dependent expression of shRNA targeting molecules essential for cell survival can be used for the generation of loss-of-function phenotypes *in vitro* and *in vivo*. This is an important prerequisite to study the functional role of genes during tumorigenesis. In addition, the observed effects of tumor growth inhibition *in vivo* as a result of Plk1 knockdown further support the use of RNAi-based strategies for cancer therapy (52) and highlight the role of Plk1 as a potent target to prevent tumorigenesis (53,54). Although the application of siRNA/Plk1 was assayed in murine studies (55) and small molecular weight inhibitors targeting Plk1 are currently being tested in clinical settings for their potential to fight neoplastic progression in humans (56), our study provides additional insight into the function of RNAi-based genomic elements, which is an important prerequisite for the generation of transgenic mouse models resting upon RNAi.

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